529 Rec'd PCT/PTO 12 JUN 2000

FORM PTO-1390 (REV 12-29-99)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 40511 Box 60

US APPLICATION NO (If known, see 37 CFR 1 5): 09/581308

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/25720	11 December 1998	12 December 1997

TITLE OF INVENTION: THERAPEUTIC USE OF LENTIVIRAL VECTORS

APPLICANT(S) FOR DO/EO/US: LUIGI NALDINI and JIN-PING SONG

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☑ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☑ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☑ Other items or information:

    International Search Report

    Written Opinion

    CRF of Sequence Listing and Statement Relating thereto

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/581308	PCT/US98/25720	40511

17. ☑ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$690.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 670.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total claims	4	- 20 =	0	X **$18.00**	$
Independent claims	1	- 3 =	0	X **$78.00**	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ **$260.00**	$
TOTAL OF ABOVE CALCULATIONS =				$ 670.00	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 670.00	
				Amount to be refunded:	$
				charged:	$

a. ☑ A check in the amount of $ 670.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-2220. A duplicate copy of this sheet is enclosed.

The Declaration/Power of Attorney, Assignment and Small Entity Declaration will be filed shortly.

Priority is claimed from U.S. Ser. No. 60/069,579 filed 12 December 1997.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

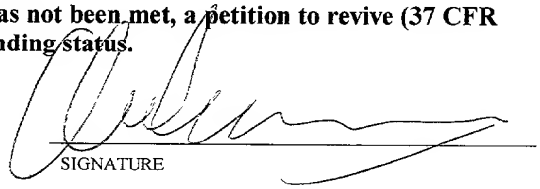
SEND ALL CORRESPONDENCE TO:

Roylance, Abrams, Berdo & Goodman, L.L.P.

1300 19th Street, N.W., Suite 600

Washington, D.C. 20036

(202) 659-9076



SIGNATURE

Dean H. Nakamura

NAME

33,981

REGISTRATION NUMBER

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/581308	PCT/US98/25720	40511

416 Rec'd PCT/PTO 12 JUN 2000

17. ☑ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $970.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $690.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 670.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total claims	4	- 20 =	0	X $18.00	$
Independent claims	1	- 3 =	0	X $78.00	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ $260.00	$

TOTAL OF ABOVE CALCULATIONS =	$ 670.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).	$
SUBTOTAL =	$
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$
TOTAL NATIONAL FEE =	$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$
TOTAL FEES ENCLOSED =	$ 670.00
Amount to be refunded:	$
charged:	$

a. ☑ A check in the amount of $ 670.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-2220. A duplicate copy of this sheet is enclosed.

The Declaration/Power of Attorney, Assignment and Small Entity Declaration will be filed shortly.

Priority is claimed from U.S. Ser. No. 60/069,579 filed 12 December 1997.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

Roylance, Abrams, Berdo & Goodman, L.L.P.

1300 19th Street, N.W., Suite 600

Washington, D.C. 20036

(202) 659-9076

SIGNATURE

Dean H. Nakamura

NAME

33,981

REGISTRATION NUMBER

THERAPEUTIC USE OF LENTIVIRAL VECTORS

FIELD OF THE INVENTION

The invention relates to the use of lentiviral vectors in the treatment of a disease resulting from or associated with a lentivirus.

BACKGROUND OF THE INVENTION

Retrovirus vectors are a common tool for gene delivery (Miller, Nature (1992) 357:455-460). The ability of retrovirus vectors to deliver an unrearranged, single copy gene into a broad range of rodent, primate and human somatic cells makes retroviral vectors well suited for transferring genes to a cell.

Lentiviruses are complex retroviruses which, in addition to the common retroviral genes gag, pol and env, contain other genes with regulatory or structural function. The higher complexity enables the lentivirus to modulate the life cycle thereof, as in the course of latent infection.

A typical lentivirus is the human immunodeficiency virus (HIV), the etiologic agent of AIDS. In vivo, HIV can infect macrophages, which are terminally differentiated cells that rarely divide. In vitro, HIV can infect primary cultures of monocyte-derived macrophages (MDM) as well as HeLa-Cd4 or T lymphoid cells arrested in the cell cycle by treatment with aphidicolin or γ irradiation.

SUMMARY OF THE INVENTION

The instant invention relates to the use of lentiviral vectors per se for a therapeutic benefit. The vector need not contain a transgene with antiviral activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts four graphs of Gag p24 antigen expression in human SupT1 lymphocytes transduced with lentiviral vector at different multiplicity of infection (M.O.I.; rectangles, triangles, ellipses) or in control non-transduced cells (lozenges) after infection with different amounts of HIV.

Figure 2 depicts Gag p24 antigen expression and cell survival after HIV infection of human primary $CD4^+$ lymphocytes transduced with either a lentiviral vector (triangles) or a murine leukemia virus based vector (squares) or non-transduced cells (diamonds).

DETAILED DESCRIPTION OF THE INVENTION

The instant invention provides use of a lentiviral vector. The vector can be one which carries a foreign gene with an anti-viral activity, however, that is not a prerequisite in the practice of the instant invention. Thus, a vector per se can be used.

The lentiviral genome and the proviral DNA have the three genes found in retroviruses: gag, pol and env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid and nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase), a protease and an integrase; and the env gene encodes viral envelope glycoproteins. The 5′ and 3′

LTR's serve to promote transcription and polyadenylation of the virion RNA's. The LTR contains all other cis-acting sequences necessary for viral replication. Lentiviruses have additional genes including vif, vpr, tat, rev, vpu, nef and vpx (in HIV-1, HIV-2 and/or SIV).

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the cis defect prevents encapsidation of genomic RNA. However, the resulting mutant remains capable of directing the synthesis of all virion proteins.

The vectors of interest are those which have an intact 5' and 3' lentivirus LTR. A vector of interest contains a packaging signal sequence comprising the leader sequence downstream of the LTR and until the beginning of the gag gene. The vector may also contain an additional portion of the gag gene to enhance packaging. The vector of interest also includes a part of the env gene containing the Rev Response Element (RRE), and it may or may not include a splice acceptor site downstream of the RRE. The vectors of interest may contain one or more transgenes, or foreign nucleic acid, and preferably a transgene with anti-viral activity. However, a vector of interest need not contain a heterologous gene.

The heterologous or foreign nucleic acid sequence, the transgene, is linked operably to a regulatory nucleic acid sequence. As used herein, the term "heterologous" nucleic acid sequence refers to a sequence that originates from a foreign species, or, if from the same species, it may be substantially modified from the original form. Alternatively, an unchanged nucleic acid sequence that is

not expressed normally in a cell is a heterologous nucleic acid sequence.

The term "operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. Preferably, the heterologous sequence is linked to a promoter, resulting in a chimeric gene. The heterologous nucleic acid sequence is preferably under control of either the viral LTR promoter-enhancer signals or of an internal promoter, and retained signals within the retroviral LTR can still bring about efficient expression of the transgene.

The foreign gene can be any transcribable nucleic acid of interest. Generally the foreign gene encodes a polypeptide. Preferably the polypeptide has some therapeutic benefit. The polypeptide may supplement deficient or nonexistent expression of an endogenous protein in a host cell. The polypeptide can confer new properties on the host cell, such as a chimeric signalling receptor, see U.S. Pat. No. 5,359,046. The artisan can determine the appropriateness of a foreign gene practicing techniques taught herein and known in the art. For example, the artisan would know whether a foreign gene is of a suitable size for encapsidation and whether the foreign gene product is expressed properly.

It may be desirable to modulate the expression of a gene regulating molecule in a cell by the introduction of a molecule by the method of the invention. The term "modulate" envisions the suppression of expression of a gene when it is over-expressed or augmentation of expression when it is under-expressed. Where a cell proliferative disorder is associated with the expression of a gene, nucleic acid sequences that interfere with the expression of a gene at the translational level can be

used. The approach can utilize, for example, antisense nucleic acid, ribozymes or triplex agents to block transcription or translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving same with a ribozyme. The target of those molecules is the lentiviral RNA. Moreover, the RNA may be a sequence of the virus not present in the vector or that has been mutated in the vector.

Antisense nucleic acids are DNA or RNA molecules which are complementary to at least a portion of a specific mRNA molecule (Weintraub, Sci. Am. (1990) 262:40). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides or more are preferred since such are synthesized easily and are less likely to cause problems than larger molecules when introduced into the target cell. The use of antisense methods to inhibit the in vitro translation of genes is well known in the art (Marcus-Sakura, Anal. Biochem. (1988) 172:289).

The antisense nucleic acid can be used to block expression of a viral protein or a dominantly active gene product, such as amyloid precursor protein that accumulates in Alzheimer's disease. Such methods are also useful for the treatment of Huntington's disease, hereditary Parkinsonism and other diseases. Antisense nucleic acids are also useful for the inhibition of expression of proteins associated with toxicity.

Use of an oligonucleotide to stall transcription can be by the mechanism known as the triplex strategy since the oligomer winds around double-helical DNA, forming a

three-strand helix. Therefore, the triplex compounds can be designed to recognize a unique site on a chosen gene (Maher et al., Antisense Res and Dev. (1991) 1(3):227; Helene, Anticancer Drug Dis. (1991) 6(6):569).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode those RNA's, it is possible to engineer molecules that recognize and cleave specific nucleotide sequences in an RNA molecule (Cech, J. Amer. Med Assn. (1988) 260:3030). A major advantage of that approach is only mRNA's with particular sequences are inactivated.

It may be desirable to transfer a nucleic acid encoding a biological response modifier. Included in that category are immunopotentiating agents including nucleic acids encoding a number of the cytokines classified as "interleukins", for example, interleukins 1 through 12. Also included in that category, although not necessarily working according to the same mechanism, are interferons, and in particular gamma interferon (γ-IFN), tumor necrosis factor (TNF) and granulocyte-macrophage colony stimulating factor (GM-CSF). It may be desirable to deliver such nucleic acids to bone marrow cells or macrophages to treat inborn enzymatic deficiencies or immune defects. Nucleic acids encoding growth factors, toxic peptides, ligands, receptors or other physiologically important proteins also can be introduced into cells. The transgene also can be an inducible toxic molecule.

The method of the invention may also be useful for neuronal, glial, fibroblast or mesenchymal cell transplantation, or "grafting", which involves transplantation of cells infected with the recombinant lentivirus of the invention ex vivo, or infection in vivo

into the central nervous system or into the ventricular cavities or subdurally onto the surface of a host brain. Such methods for grafting will be known to those skilled in the art and are described in Neural Grafting in the Mammalian CNS, Bjorklund & Stenevi, eds. (1985).

For diseases due to deficiency of a protein product, gene transfer could introduce a normal gene into the affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For example, it may be desirable to insert a Factor VIII or IX encoding nucleic acid into a lentivirus for infection of a muscle, spleen or liver cell.

The promoter sequence may be homologous or heterologous to the desired gene sequence. A wide range of promoters may be utilized, including a viral or a mammalian promoter, and an inducible promoter. Cell or tissue specific promoters can be utilized to target expression of gene sequences in specific cell populations. Suitable mammalian and viral promoters for the instant invention are available in the art.

Optionally during the cloning stage, the nucleic acid construct referred to as the transfer vector, having the packaging signal and the heterologous cloning site, also contains a selectable marker gene. Marker genes are utilized to assay for the presence of the vector, and thus, to confirm infection and integration. The presence of a marker gene ensures the selection and growth of only those host cells which express the inserts. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, e.g., histidinol, puromycin, hygromycin, neomycin, methotrexate etc. and cell surface markers.

The recombinant virus of the invention is capable of

transferring a nucleic acid sequence into a mammalian cell. The term, "nucleic acid sequence", refers to any nucleic acid molecule, preferably DNA, as discussed in detail herein. The nucleic acid molecule may be derived from a variety of sources, including DNA, cDNA, synthetic DNA, RNA or combinations thereof. Such nucleic acid sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, poly A sequences or other associated sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from cells and used to produce cDNA by reverse transcription or other means.

Preferably, the recombinant lentivirus produced by the method of the invention is a derivative of human immunodeficiency virus (HIV).

The vectors of interests are produced using known methods. The vectors of interest can be introduced into cells either as the nucleic acid or encapsidated as a virus particle. An artisan is familiar with methods for encapsidating a lentiviral vector of interest. The vectors are introduced into target cells using methods known by those of skill in the art.

Thus, the vectors can be introduced into human cell lines by calcium phosphate transfection, lipofection or electroporation, generally together with a dominant selectable marker, such as neo, DHFR, Gln synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones. The selectable marker gene can be the transgene.

A likely means for transforming host cells with a vector of interest is by infecting cells with virus

particles carrying a vector of interest. Thus, the vector of interest would be encapsidated using known packaging systems, such as that taught in U.S. Pat. No. 5,686,279 and in Naldini et al. Science (1996) 272:263-267. Briefly, using either a stable packaging cell line or by transient transfection, the vector of interest is introduced into a cell which packages the vector of interest into viral particles. The virus particles are obtained from the culture medium, treated as known in the art to provide a virus preparation.

The target cell then is exposed to the virus preparation. That can be via in vivo administration means, wherein the virus preparation is administered to a host, for example, in a parenteral form. Alternatively, cells from the host can be retrieved and maintained in culture where those cells are exposed to the virus preparation. Once transformed, stably or not, the cells then can be returned to the host.

While the therapeutic benefit of the instant invention can be obtained by the vector per se, it is preferred that the vector carry a transgene. Preferably that transgene is one which itself has a therapeutic effect. Thus, the vectors of interest should have a place in current therapy of diseases associated with lentivirus.

Although the techniques used to construct vectors and the like are provided in standard resource materials which describe specific conditions and procedures, for convenience, the following paragraphs may serve as a guideline.

Construction of the vectors of the invention employs standard ligation and restriction techniques which are well understood in the art (see Maniatis et al., in Molecular Cloning: A Laboratory Manual, Cold Spring

Harbor Laboratory, N.Y., 1982). Isolated plasmids, DNA sequences or synthesized oligonucleotides are cleaved, tailored and religated in the form desired.

Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are understood in the art, and the particulars of which are specified by the manufacturer of the commercially available restriction enzymes, see, e.g. New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequences is cleaved by one unit of enzyme in about 20 μl of buffer solution. Typically, an excess of restriction enzyme is used to ensure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, which may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods of Enzymology 65:499-560 (1980).

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTP's) using incubation times of about 15 to 25 minutes at 20°C in 50 mM Tris (pH 7.6) 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 5-10 μM dNTP's. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTP's are present. If desired, selective repair can be performed by supplying only one of the dNTP's, or with selected dNTP's, within the limitations dictated by the nature of the sticky ends. After treatment with

Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or Bal-31 results in hydrolysis of any single-stranded portion.

Ligations can be performed in 15-50 μl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 mg/ml BSA, 10 mM-50 mM NaCl and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μg/ml total DNA concentrations (5-100 mM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 μM total ends concentration.

Lentiviral vectors (Naldini et al., supra and Proc. Natl. Acad. Sci. (1996) 93:11382-11388) have been used to infect human cells growth-arrested in vitro and to transduce neurons after direct injection into the brain of adult rats. The vector was efficient at transferring marker genes in vivo into the neurons and long term expression in the absence of detectable pathology was achieved. Animals analyzed ten months after a single injection of the vector, the longest time tested so far, showed no decrease in the average level of transgene expression and no sign of tissue pathology or immune reaction. (Blomer et al., J. Virol. (1997) 71:6641-6649). An improved version of the lentiviral vector in which the HIV virulence genes env, vif, vpr, vpu and nef were deleted without compromising the ability of the vector to transduce non-dividing cells have been developed. The multiply attenuated version represents a substantial improvement in the biosafety of the vector (Zufferey et al. Nat. Biotech. (1997) 15:871-875).

Viral supernatants are harvested using standard techniques such as filtration of supernatants 48 hours post transfection. The viral titer is determined by infection of, for example, 10^6 NIH 3T3 cells or 10^5 HeLa cells with an appropriate amount of viral supernatant, in the presence of 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO). Forty-eight hours later, the transduction efficiency is assayed.

While not wanting to be bound to any posited hypothesis, it is believed the mechanism of the resistance was mapped to a post-integration step and shown to be dependent on an intact HIV LTR in the vector. On HIV infection of transduced cells, transcription from the vector LTR was enhanced greatly, resulting in increased expression of the transgene. Conceivably the vector RNA competes effectively with the viral RNA's both for binding the transactivators and for packaging by the budding viral particles, resulting in inhibition of viral replication and mobilization and spreading of the vector. Viral particles collected from the infected transduced cells were less infectious than virus collected from infected non-transduced cells, and transferred efficiently the transgene into naive cells.

Thus, expression of both the vector and the virus in the same cell is detrimental to viral replication, and result in mobilization and spreading of the transgene into selected target cells of HIV. That effect and the strong enhancement of transgene expression induced by HIV are significant advantages of an HIV-derived vector of anti-HIV gene therapy applications.

Thus, the instant vector will find use alone, either containing a transgene or not, and preferably the transgene has an antiviral activity; or in combination with another vector carrying a transgene with antiviral

activity, wherein the instant vector does or does not contain a transgene.

The viral particles can be further purified from the viral supernatants as known in the art.

The viral particles or vector nucleic acid can be administered to a host with a disorder associated with or caused by a lentivirus using known techniques.

Actual delivery of the vectors or particles is accomplished by using any physical method that will transport same into a host and into the target cell. As used herein, "vector", means both a bare recombinant lentiviral vector and recombinant lentiviral particle. Simply dissolving a vector in Hanks' balanced saline solution or phosphate buffered saline is sufficient to provide a solution useful for injection. There are no known restrictions on the carriers or other components that can be coadministered with the vector (although compositions that degrade the virion or polynucleotides thereof should be avoided in the normal manner with vectors).

Pharmaceutical compositions can be prepared as injectable formulations to be delivered intramuscularly, including implantable pumps (known by those of skill in the art and described, for example, in U.S. Pat. No. 5,474,552). Numerous formulations for injection are known and can be used in the practice of the instant invention. The vectors can be used with any pharmaceutically acceptable carrier for ease of administration and handling.

Such aqueous solutions can be buffered, if desired, and the liquid diluent first rendered isotonic with saline or glucose. Solutions of the vector as a free acid (DNA

contains acidic phosphate groups) or a pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. A dispersion of viral particles also can be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of storage and use, the preparations contain a preservative to prevent the growth of microorganisms. The sterile aqueous media employed are obtainable by standard techniques well-known to those skilled in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that administration by a syringe is possible. The formulation must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the vector in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze drying which yield a powder of the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

The therapeutic compounds of this invention may be administered to a host alone or in combination with pharmaceutically acceptable carriers. As noted above, the relative proportions of active ingredient and carrier are determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice.

The dosage of the instant therapeutic agents which will be most suitable for prophylaxis or treatment will vary with the form of administration, the particular recombinant vector chosen and the physiological characteristics of the particular patient under treatment. Generally, small dosages will be used initially and, if necessary, will be increased by small increments until the optimum effect under the circumstances is reached. Exemplary dosages are within the range of 10^8 up to approximately 5 x 10^{15} particles in a total volume of 3-10 ml.

The invention now having been described in detail, provided hereinbelow are non-limiting examples

demonstrating various embodiments of the instant invention.

Example 1

CONSTRUCTION OF THE LENTIVIRAL VECTORS

The lentiviral transfer vector plasmids were derived from the plasmid pHR'-CMV-LacZ described previously in Naldini et al., Science (1996) 272:263-267. Plasmid pHR'-CMV-Neo was derived by substituting the BamHI-XhoI fragment of pHR'-CMV-LacZ containing the E.coli LacZ gene with a BamHI-XhoI fragment containing the neomycin phosphotransferase gene of E.coli (Beck et al., Gene (1982) 19:327-336).

pHR2 is a lentiviral transfer vector in which 124 base pairs (bp) of nef sequences upstream of the 3' LTR in pHR have been replaced with a polylinker both to reduce HIV-1 sequences and to facilitate transgene cloning. pHR2 was derived from pHR'-CMV-LacZ by replacing the 4.6 kilobase (kb) ClaI-StuI fragment with an 828 bp ClaI-StuI fragment generated by PCR using pHR'-CMV-LacZ as the template and with the oligonucleotide primer, 5'-CCATCGATCACGAGACTAGTCCTACGTATCCCCGGGGACGGGATCCGCGGAATTCC GTTTAAGAC-3' (SEQ ID NO:_____) and the primer 5'-TTATAATGTCAAGGCCTCTC-3' (SEQ ID NO:_____) in a three part ligation with a 4.4 kb StuI-NcoI fragment and a 4.5 kb NcoI-ClaI fragment from pHR'-CMV-LacZ.

Plasmid pHR2-PGK-GFP was derived by cloning the XhoI-BamHI fragment of pRT43.3PGKF3 (WO 97/07225) containing the human PGK promoter (GenBank Accession number #M11958 nucleotides 2-516) and the BamHI-NotI fragment of plasmid of pEGFP1 (Clontech) containing a codon usage-optimized and improved version of the Green Fluorescent Protein (GFP) of A. victoria and a NotI-SacII linker, into the Xhol and SacII sites of pHR2.

Example 2

INHIBITION OF HIV-1 REPLICATION OF LYMPHOCYTES TRANSDUCED BY THE LENTIVIRAL VECTOR

Human SupT1 T-lymphoblastoid cells were obtained by ATCC. Human $CD4^+$ primary blood lymphocytes (PBL) were separated from buffy coats from donations, stimulated with 2.5 μg/ml phytohemagglutinin or Dynal beads coated with OKT3 and CD28 antibodies for 2 days, then washed and cultured with 100 U/ml of interleukin 2 (Chiron) in AIM-V medium (Gibco). The SupT1 cells or PBL were transduced either with lentivector or a murine leukemia virus (MLV) vector carrying the same transgene overnight in the presence of 2 μg/ml polybrene, then washed and selected for transgene expression after 48 hrs.

All vectors were produced by transient transection of 293T cells and pseudotyped with the VSV.G envelope as described previously (Naldini et al., Proc. Natl. Acad. Sci. (1996) 93:11382-88). Cells transduced with vectors carrying the neomycin resistance gene were selected in medium containing 1 mg/ml G418, then cultured in normal medium for virus challenge. Cells transduced with vectors carrying the green fluorescent protein (GFP) as transgene were selected by cell sorting.

The cells were challenged with increasing amounts of HIV virus. HIV-1 virions were produced either by 293T cells transfected with the proviral infectious molecular clone R8, or by SupT1 cells chronically infected with R8 virus. R8 is a lymphocytotropic HIV-1 hybrid of the HXB2-D and NL43 isolates that expresses all HIV reading frames (Gallay et al., Cell (1995) 83:569-576). The virus stock was titered on HeLa P4 cells and had an infectivity of 1,000 to 3,000 infectious units/ng p24. The cells were washed twice after overnight incubation with the virus in the presence of 2 μg/ml polybrene, and further cultured

for up to 3 weeks. Every 3-4 days, the conditioned medium was collected and HIV replication was determined by accumulation of HIV-1 Gag p24 in the medium by a commercially available ELISA kit (DuPont).

In the first experiment (see Figure 1), SupT1 cells transduced by lentiviral vector carrying the neomycin resistance gene, pHR'-CMV-Neo, were tested. HIV accumulated in control non-transduced cultures. On the other hand, in cells transduced by the lentiviral vector, pHR2, HIV replication was detected only for the higher amounts of HIV and p24 accumulation was decreased dramatically and delayed. Similar results were obtained with three different SupT1 populations selected after transduction with the lentiviral vector at different multiplicity of infection (M.O.I.). Moreover, no cytopathic effect was observed in lentivector transduced cells infected with up to 10 ng of HIV. In contrast, the non-transduced cultures developed cytopathic effect with all tested amounts of HIV.

The applicability of the inhibitory effect on HIV growth to primary cells and its specificity for lentiviral vectors were tested in another experiment (see Figure 2). $CD4^+$ PBL's were transduced with either lentivector (pHR2-PGK-GFP) or the MLV retrovector carrying the same GFP transgene driven by the human PGK promoter, and sorted for transgene expression. The selected populations then were challenged with HIV virus as described above. Both the non-transduced cells (indicated in the figure by diamonds) and sorted cells transduced by the MLV retrovector (indicated by squares) yielded similar levels of p24 antigen in the culture medium. However, the cells transduced by the lentiviral (indicated by triangles) yielded sharply reduced p24 even after inoculation with high doses of HIV (100 ng p24 equivalent of virus). Moreover, there were twice as many cells transduced by the

lentivector surviving 13 days after infection than those transduced by the retrovector or non-transduced. In cells transduced by the lentivector, transgene expression was augmented significantly after infection with the HIV virus.

All publications and patent applications cited in this specification are herein incorporated by reference in their entirety as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

As will be apparent to those skilled in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed above, for example to transfect and transduce other mammalian cell types, without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described above, are, therefore, to be considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples contained in the foregoing description.

We claim:

1. A method for treating a host infected with a lentivirus comprising exposing said host to a lentivirus vector and a biologically acceptable carrier, excipient and diluent.

2. The method of claim 1, wherein said vector has an intact 5′ LTR.

3. The method of claim 1, wherein said lentivirus is human immunodeficiency virus (HIV).

4. The method of claim 3, wherein said HIV is HIV-1.

FIG. 1

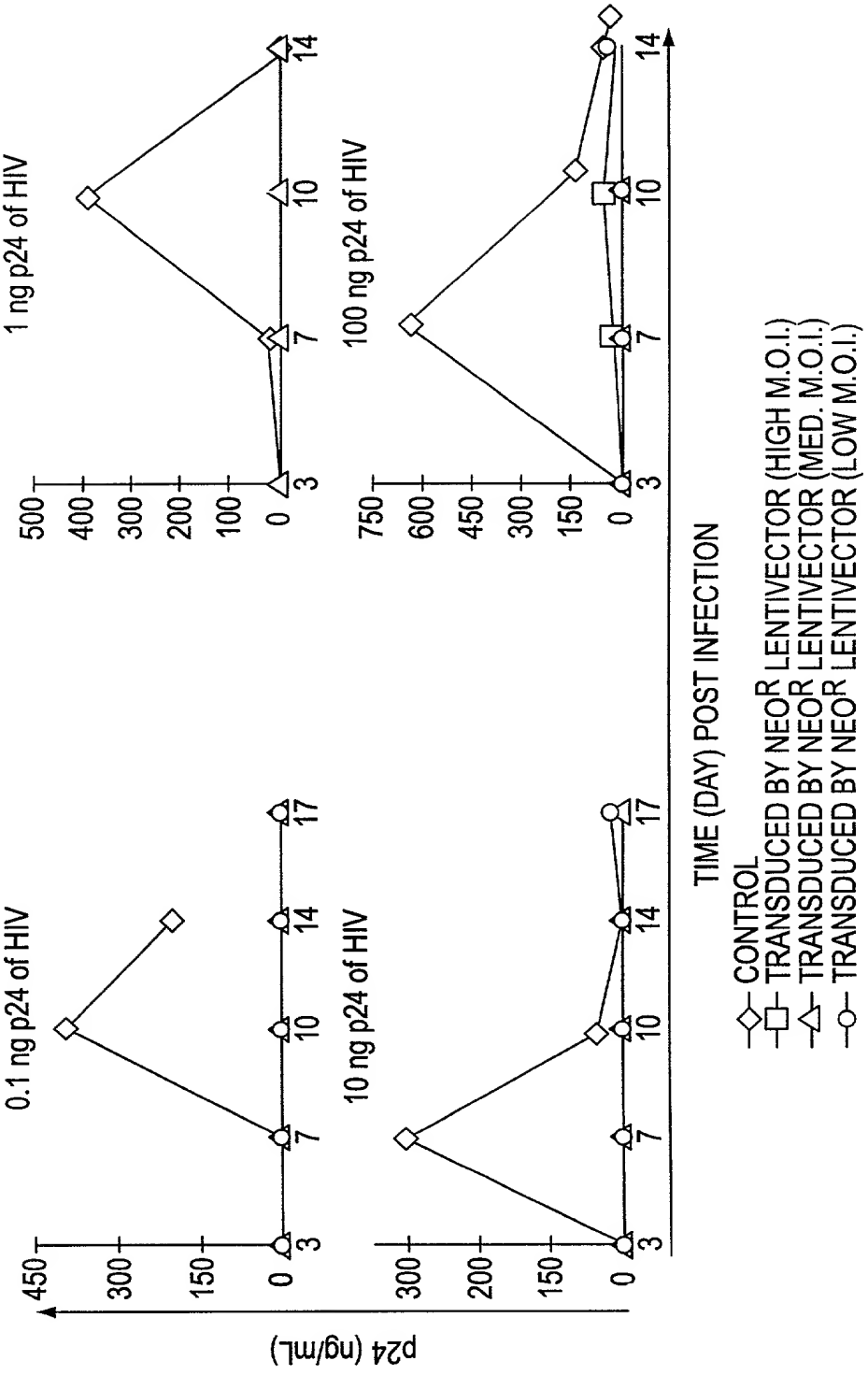
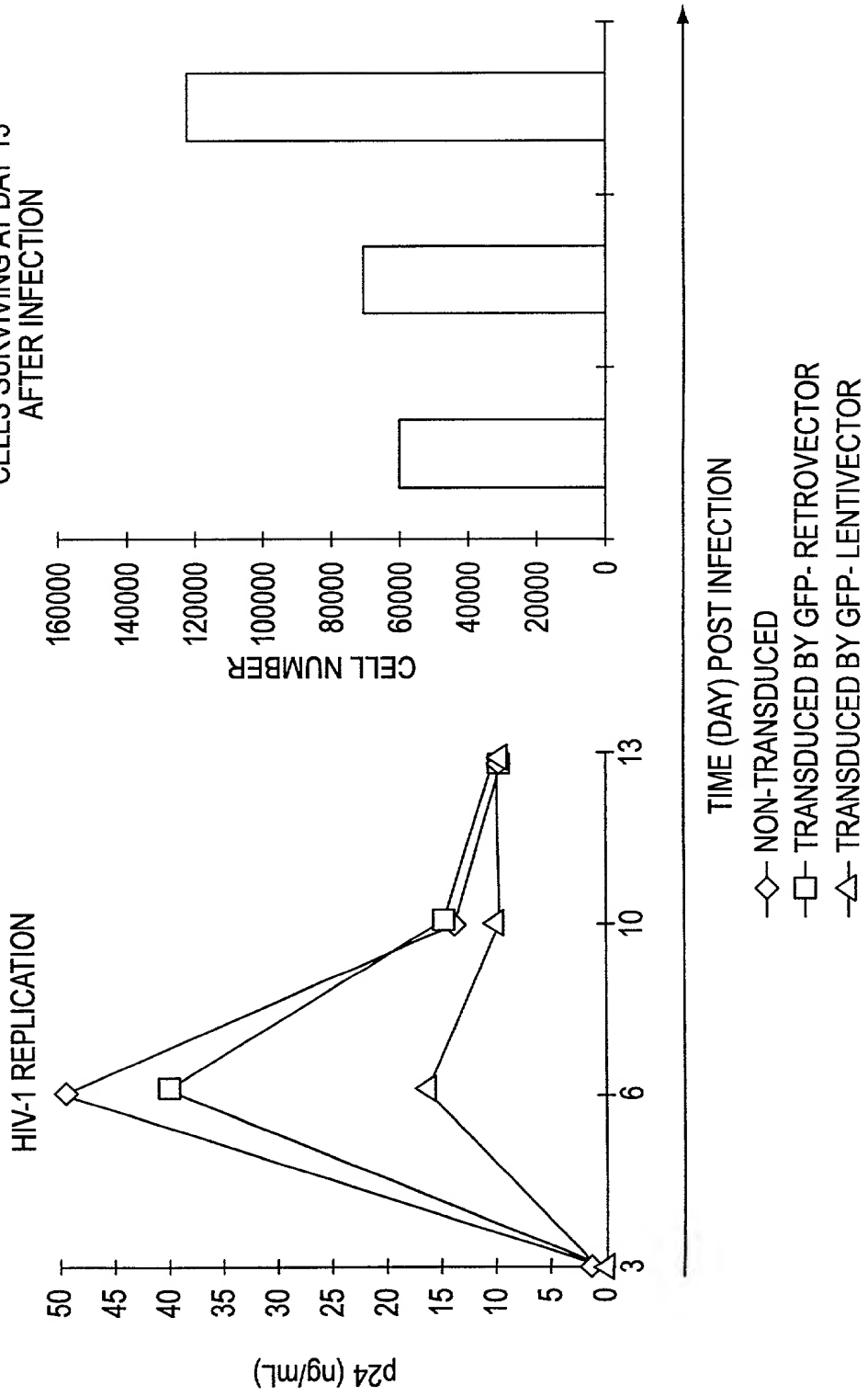


FIG. 2



DECLARATION /POWER OF ATTORNEY FOR PATENT APPLICATION JOINT INVENTORS

As the named inventors, we hereby declare:

Our residences, post office addresses and citizenships are as stated next to our names below:

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled THERAPEUTIC USE OF LENTIVIRAL VECTORS, the specification of which was filed on 12 June 2000 as application U.S. Serial No. 09/581,308.

We hereby state that we reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the patentability of the application in accordance with Title 37, Code of Federal Regulation, §1.56.

Prior Foreign Applications

We hereby claim priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below. We have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is based.

Country	Application Number	Date of Filing (day, month, year)	Date of Issue (day, month, year)	Priority Claimed Under 35 U.S.C. §119
United States	60/069,879	12 December 1997		Yes x No ☐
WIPO	US9825720	11 December 1998		Yes x No ☐

Power of Attorney

We hereby appoint, both jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected herewith as well as before any office or agency of a foreign county or any international organization in connection with any foreign counterpart application claiming priority to this application, including the power to appoint agents and local representatives in connection with such foreign applications, the following attorneys of Roylance, Abrams, Berdo & Goodman, their registration numbers being listed after their names:

DAVID S. ABRAMS	REG. NO. 22,576	DARYL BASHAM	REG. NO. 45,869
ROBERT H. BERDO	REG. NO. 19,415		
ALFRED N. GOODMAN	REG. NO. 26,458		
MARK S. BICKS	REG. NO. 28,770		
JOHN E. HOLMES	REG. NO. 29,392		
LANCE G. JOHNSON	REG. NO. 32,531		
DEAN H. NAKAMURA	REG. NO. 33,981		
GARRETT V. DAVIS	REG. NO. 37,023		
STACEY LONGANECKER	REG. NO. 33,952		
JOSEPH J. BUCZYNSKI	REG. NO. 35,084		

U.S. Ser. No. 09/581,308
Declaration/Power of Attorney

All correspondence and telephone communications should be addressed to:

Dean H. Nakamura
Roylance, Abrams, Berdo & Goodman, L.L.P.
1300 19th Street, N.W. Suite 600
Washington, D.C. 20036
Telephone: (202) 659-9076
Facsimile: (202) 659-9344

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information knowledge and belief are believed to be true; and further that these were made with the knowledge that false statements made willfully are punishable by fine, imprisonment, or both a fine and imprisonment under Section 1001 of Title 18 of the United States; and further that false statements made willfully may jeopardize the validity of any patent issuing on an application in which the false statements were made.

Signature _____ Date 2/7/2001

Full Name of
1st Inventor Naldini (Family Name) Luigi (Given Name)

Residence Torino, Italy ITX

Citizenship Italy

Post Office
Address Corso Lepanto 12 10134 Torino Italy

1-0[illegible]

Signature _____ Date 2/9/2001

Full Name of
2nd Inventor Song (Family Name) Jia-Ping (Given Name)

2-0[illegible]

Residence Palo Alto, California CA

Citizenship China

Post Office
Address 3455 Rainbow Drive, Palo Alto, CA 94306

Signature _____ Date 2/9/2001

SEQUENCE LISTING

<110> Song, Jin-Ping
Naldini, Luigi
Cell Genesys

<120> THERAPEUTIC USE OF LENTIVIRAL VECTORS

<130> F126422

<140>
<141>

<150> 60/069,579
<151> 1997-12-12

<160> 2

<170> PatentIn Ver. 2.0

<210> 1
<211> 65
<212> DNA
<213> primer

<400> 1
ccatcgatca cgagactagt cctacgtatc cccggggacg ggatccgcgg aattccgttt 60
aagac 65

<210> 2
<211> 20
<212> DNA
<213> primer

<400> 2
ttataatgtc aaggcctctc 20